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Practical Synthetic Route to Functionalized Rhodamine Dyes

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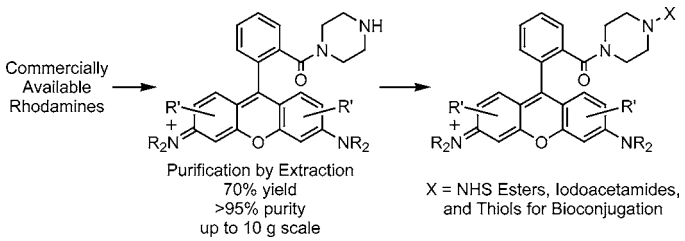
ABSTRACT



An efficient method for the synthesis of functionalized rhodamine derivatives has been developed. Multigram quantities of these water-soluble fluorophores can be prepared from inexpensive precursors and purified without the use of chromatography. A series of protein-reactive functional groups has been installed through subsequent reactions, providing materials for biomolecule modification. For multicolor applications, a solid-phase purification strategy has been developed to afford rhodamine derivatives possessing a wide range of spectral properties.

The utility of fluorescent dyes spans many scientific disciplines. In biology, fluorescent probes have been used extensively to track the locations of proteins in living cells,[1] to detect specific protein functional groups,[2] and to measure intracellular ion concentrations.[3] More recently, fluorescence resonance energy transfer (FRET) has become a powerful tool for measuring distance relationships in biomolecular assemblies.[4] In physics, fluorescent dyes are essential components of many lasers, and in materials science, fluorescent compounds have been used to create light-harvesting materials[5] and small-molecule sensors.[6]

Due to this utility, a variety of dye molecules have been appended with reactive functional handles for further conjugation. However, the extremely high cost of these compounds from commercial sources (typically >$30 000/g for isomerically pure dyes) generally precludes their use in materials science applications. In contrast, the unfunctionalized analogues of these dyes are often available at low cost (<$1/g), providing more economical precursors to reactive chromophores if efficient functionalization chemistry can be developed. For this purpose, we report herein a general synthetic method for the direct modification of commercially available rhodamines, affording multigram quantities of water-soluble fluorescent dyes that can be conjugated to virtually any substrate of interest. This method has been carried out on several rhodamine derivatives, thus offering

(1) For a general review, see: *Fluorescent and Luminescent Probes for Biological Activity*; Mason, W. T., Ed.; Academic Press: San Diego, 1999.

(2) (a) Griffin, B. A.; Adams, S. R.; Tsien, R. Y. *Science* **1998**, *281*, 269−271. (b) Adams, S. R.; Campbell, R. E.; Gross, L. A.; Martin, B. R.; Walkup, G. K.; Yao, Y.; Llopis, J.; Tsien, R. Y. *J. Am. Chem. Soc*. **2002**, *124*, 6063. (c) Lemieux, G. A.; de Graffenried, C. L.; Bertozzi, C. R. *J. Am. Chem. Soc*. **2003**, *125*, 4708.

(3) (a) Minta, A.; Kao, J. P. Y.; Tsien, R. Y. *J. Biol. Chem.* **1989**, *264*, 8171. (b) Minta, A.; Tsien, R. Y. *J. Biol. Chem.* **1989**, *264*, 19449.

(4) (a) Heyduk, T. *Curr. Opin. Biotechnol.* **2002**, *13*, 292. (b) Ha, T.; Zhuang, X.; Babcock, H.; Kim, H.; Orr, J. W.; Williamson, J. R.; Bartley, L.; Russell, R.; Herschlag, D.; Chu, S. *The Study of Single Biomolecules with Fluorescence Methods*: *Springer Series in Chemical Physics*; Springer Publishing: New York, 2001; Vol. 67, pp 326−337.

(5) (a) Adronov, A.; Fréchet, J. M. J. *Chem. Commun*. **2000**, 1701. (b) Hecht, S.; Fréchet, J. M. J. *J. Am. Chem. Soc*. **2001**, *123*, 6959. (c) Serin, J. M.; Brousmiche, D. W.; Fréchet, J. M. J. *J. Am. Chem. Soc*. **2002**, *124*, 11848.

(6) (a) Czarnik, A. W. *Fluorescent Chemosensors for Ion and Molecular Recognition*; American Chemical Society: Washington, DC, 1993. (b) Zhang, S.-W.; Swager, T. M. *J. Am. Chem. Soc.* **2003**, *125*, 3420.

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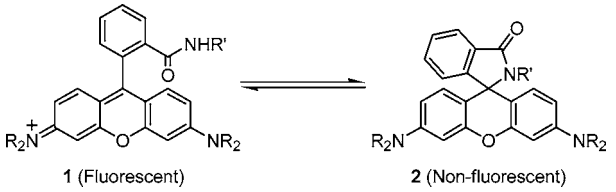
a convenient series of compounds for multicolor fluorescence applications.

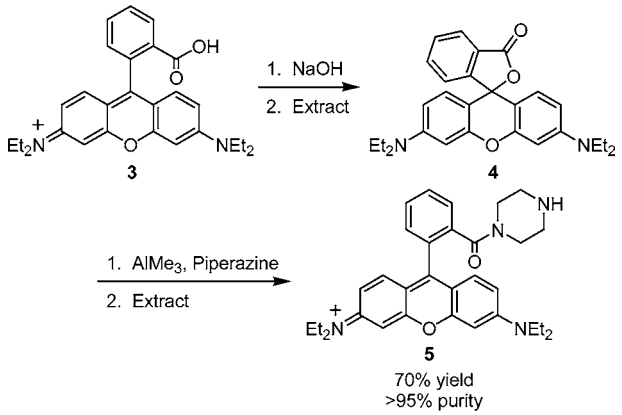
As the common condensation route[7] used to prepare xanthene dyes introduces a carbonyl group in the 2′ position, this location has been targeted previously for modification through amide bond formation.[8] However, it has been observed that secondary amides of rhodamines, such as **1**, rapidly cyclize to form nonfluorescent lactams under all but the most acidic conditions, Scheme 1. This prevents the use of these compounds for most biological labeling experiments.[9]

Scheme 1. Cyclization of Rhodamine Amides



As a simple way to avoid this cyclization pathway, we have developed a convenient method for the preparation of tertiary amide **5** from rhodamine B (**3**).[10] Exposure of lactone **4** (which is commercially available or can be prepared in quantitative yield from **3**) to 4 equiv of piperazine and 2 equiv of $AlMe_3$ in refluxing CH_2Cl_2 results in clean conversion to tertiary amide **5**, Scheme 2.[11] We have found these

Scheme 2. Synthesis of Rhodamine-Tertiary Amide Derivatives



conditions to be uniquely effective for this transformation, as numerous other procedures employing Lewis Acids and carbodiimide coupling agents failed to produce appreciable yields of the desired amide or led to the formation of unidentified side products.

After quenching the aluminum salts, compound **5** can be purified using a simple workup procedure. Due to the high water solubility of **5**, lactone **4** can be extracted selectively from basic aqueous solution with EtOAc. After acidification and saturation with NaCl, **5** can then be extracted from the aqueous layer with $^{i}PrOH/CH_2Cl_2$ (2:1). This procedure routinely affords multigram quantities of **5** in 70% overall yield and >95% purity (as determined by ^1H NMR, MALDI-MS, and HPLC, Figure 1). For most applications, this material requires no further purification. Upon conjugation, the absorption and emission spectra **5** of are red-shifted by 10 nm, relative to compound **3**, Figure 1c. This material is highly soluble in aqueous solution and retains its fluorescence emission under a wide range of pH conditions (Figure 2).

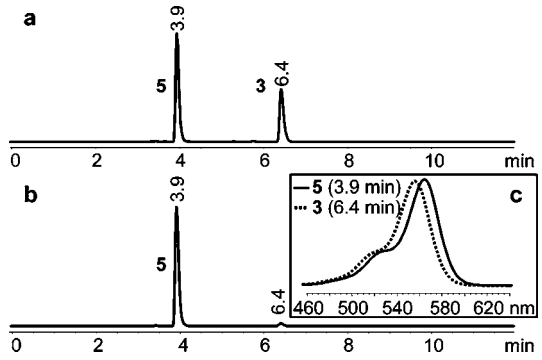


Figure 1. Purification of rhodamine **5**. Reversed-phase HPLC analysis (monitored at 565 nm) of the reaction mixture (a) before workup and (b) after extraction. HPLC and ^1H NMR analysis indicate >95% purity. (c) UV/vis spectra of **3** and **5**.

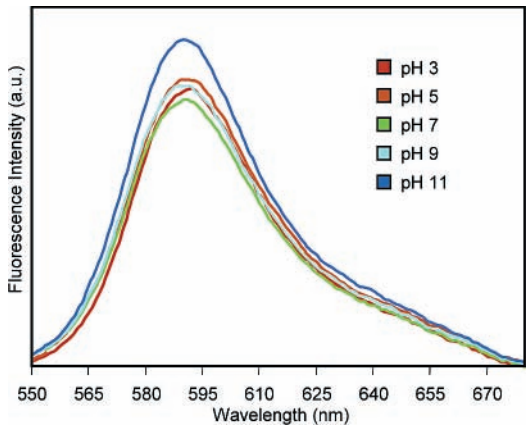


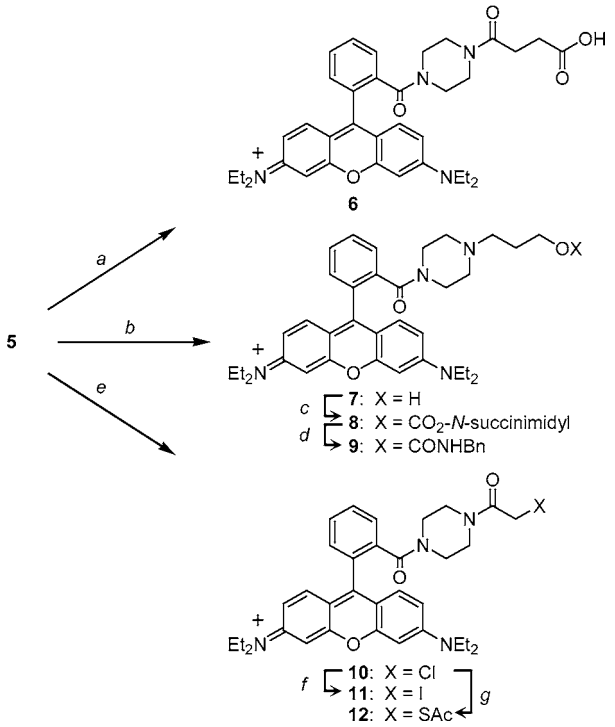
Figure 2. Fluorescence emission of **5** as a function of pH. Each sample consists of 2.5 μM **5** in 5 mM phosphate buffer adjusted to the appropriate pH with HCl or NaOH.

(7) Scala-Valero, C.; Doizi, D.; Guillaumet, G. *Tetrahedron Lett.* **1999**, *40*, 4803 and references therein.

(8) (a) Adamczyk, M.; Grote, J. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1539. (b) Adamczyk, M.; Grote, J. *Synth. Commun.* **2001**, *31*, 2681.

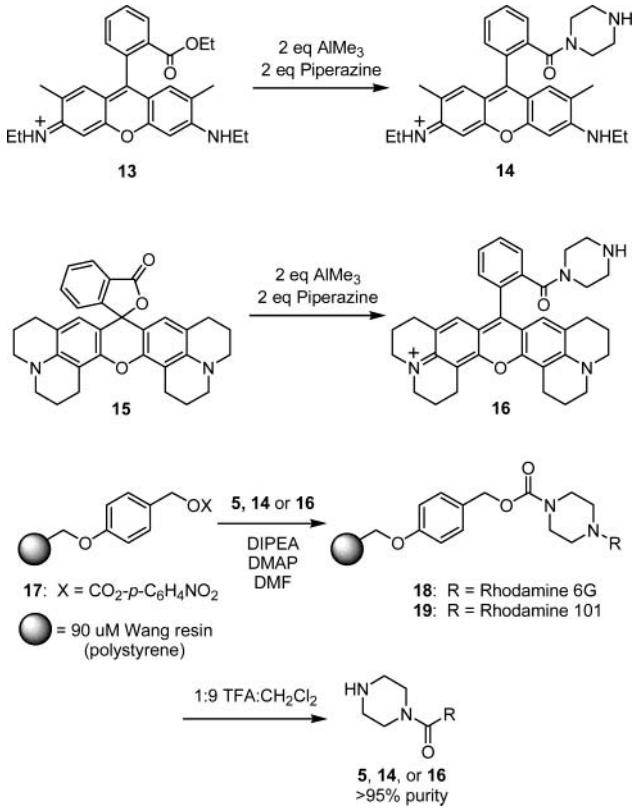
(9) An elegant solution to this problem has been provided by combining fluorescein and rhodamine chromophores in a single fluorescent probe. The resulting compound fluoresces at all pH values, although significant changes in the emission spectra are observed. Adamczyk, M.; Grote, J. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2327.

Scheme 3. Rhodamine Derivatives for Protein Functionalization[a]



[a] Reaction conditions: (a) Succinic anhydride, Et_3N, DMAP, CH_2Cl_2, 72%. (b) 3-Bromopropanol, DIPEA, DMF, 71%. (c) *N*,*N*′-Disuccinimidyl carbonate, pyridine. (d) $BnNH_2$, pyridine/H_2O, 87% from **7**. (e) Chloroacetyl chloride, pyridine, CH_2Cl_2, 95%. (f) NaI, MeOH, acetone, 88%. (g) NaSAc, DMF, 64%.

Scheme 4. Synthesis of Rhodamine Derivatives for Multicolor Labeling Applications



The secondary amine of **5** can be further elaborated to provide common functional groups used in bioconjugation reactions. For lysine modification, acid **6** can be prepared through exposure to succinic anhydride, NEt_3, and DMAP, Scheme 3. A variety of functionality can be introduced through alkylation of the amine, as exemplified by **7**. The hydroxyl group of this derivative can be transformed readily to NHS-carbonate **8**, which has been coupled to both small-molecule amines and proteins (see Supporting Information for a protein labeling protocol). Cysteine-reactive compounds can be prepared through acylation of the amine with chloroacetyl chloride, followed by conversion to iodoacetamide **11** using Finkelstein conditions. Chloroacetamide **10** can also be converted to thioacetate **12** using a similar protocol. All of these derivatives can be obtained as pure compounds without the use of chromatography. The low cost, high purity, and excellent spectral properties of these materials provide attractive alternatives for the modification of a range of protein reactive groups.

The piperazine coupling protocol described above has also proven to be effective for other rhodamine substrates. We have found that both rhodamine 6G (**13**) and rhodamine 101 (**15**) react cleanly under these conditions to afford piperazine derivatives **14** and **16**, respectively, Scheme 4. However, these reactions generally proceed with lower levels of conversion, and although the starting materials for these reactions are similarly inexpensive, the unreacted dyes are difficult to remove. Extractions have been ineffective for the purification of these derivatives due to solubility changes of the dyes, and while silica gel chromatography can be used to isolate these compounds, the high polarity of the dyes renders this process difficult and low yielding.

As an alternative purification strategy, we have found that the piperazine derivatives can be isolated using a solid-phase capture strategy. After the aluminum salts are quenched, the reaction mixtures are exposed to nitrophenyl carbonate-functionalized[12] Wang resin (polystyrene functionalized with 4-benzyloxybenzyl alcohol), Scheme 4. As shown in Figure 3a,b, this exposure selectively removes the piperazine derivative from solution. After thorough rinsing, the desired chromophore can be released from the dark-red beads through exposure to a TFA/CH_2Cl_2 (1:9) mixture. HPLC

(10) Successful formation of rhodamine tertiary amides using TBTU as a coupling agent has been reported: (a) Torneiro, M.; Still, W. C. *J. Am. Chem. Soc.* **1995**, *117*, 5887. (b) Torneiro, M.; Still, W. C. *Tetrahedron* **1997**, *53*, 8739. Other approaches have also been described in the patent literature; see: (c) Haugland, R. P.; Singer, V. L.; Yue, S. T. US Patent 6,399,392. Mayer, U.; Oberlinner, A. US Patent 4,647,675. For an example using fluoresceins, see: (d) Gao, J.; Wang, P.; Giese, R. *Anal. Chem.* **2002**, *74*, 6397.

(11) Basha, A.; Lipton, M.; Weinreb, S. M. *Tetrahedron Lett.* **1977**, *48*, 4171.

(12) Francis, M. B.; Jacobsen, E. N. *Angew. Chem., Int. Ed.* **1999**, *38*, 937.

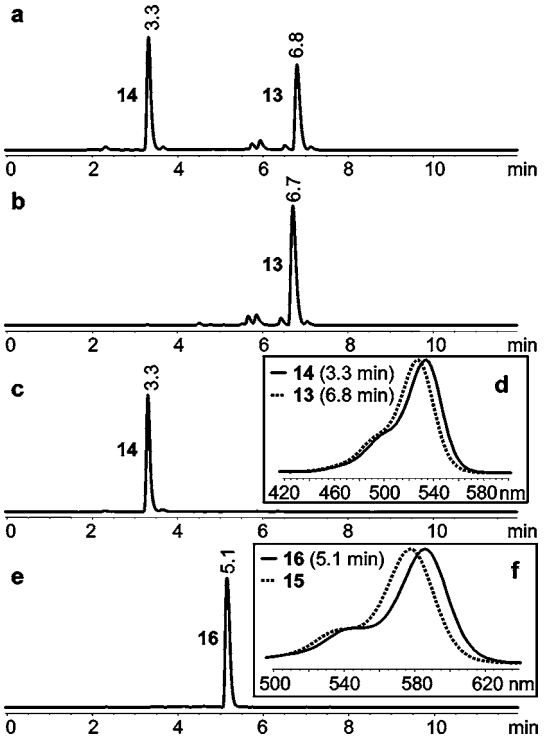


Figure 3. Purification of rhodamines **14** and **16** using a resin-capture strategy. (a) Reversed-phase HPLC analysis of the crude reaction mixture for **13**. Compound **14** can be detected at 3.3 min. (b) HPLC analysis of the supernatant after exposure to solid-phase resin **17**. Compound **14** has been completely removed from the solution. (c) HPLC analysis of the supernatant after exposing resin **18** to TFA/CH_2Cl_2 (1:9). HPLC and NMR analysis indicate >95% purity for compound **14**. (d) UV/vis spectra of **13** and **14**. (e) HPLC analysis of the supernatant after exposing resin **19** to TFA/CH_2Cl_2 (1:9). HPLC and NMR analysis indicate >95% purity for compound **16**. (f) UV/vis spectra of **15** and **16**.

analysis of the material obtained using this method indicates exceptionally high purity, Figure 3c,e. While this isolation procedure is less suited for multigram applications, it can nonetheless provide 100−200 mg quantities of highly pure dye per gram of resin. Through additional experiments, we have found that piperazine derivatives **14** and **16** can be converted to compounds for protein conjugation in a fashion similar to compound **5**.

Although all of these dyes exhibit strong fluorescence in aqueous buffer, conjugation to the piperazine moiety alters the optical properties in somewhat unpredictable ways. Lower extinction coefficients were observed for **14** and **16** (Figure 4) relative to that observed for **5**. The quantum yield

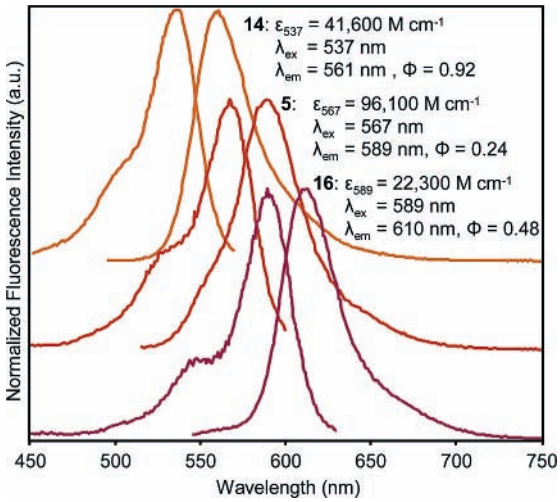


Figure 4. Spectral properties of **5**, **14**, and **16**. Quantum yields were determined as described in Supporting Information.

of **14** resembles that of unmodified rhodamine 6G, but 30 and 38% reductions are observed for **5** and **16**, respectively.[13] Nonetheless, it is anticipated that the high degree of spectral overlap between **5**, **14**, and **16** will provide useful pairs of chromophores for FRET applications in biology and for the construction of light-harvesting systems.

In summary, we have reported an efficient method for the conversion of inexpensive rhodamine derivatives into readily functionalizable chromophores for biological labeling and materials science applications. For single-color applications, rhodamine B derivatives can be easily prepared on a multigram scale. Using a solid-phase purification strategy, additional rhodamine derivatives can be prepared to access a range of spectral properties.

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Supporting Information Available: Full experimental procedures and characterization data for compounds **5**−**12**, **14**, and **16**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(13) See Supporting Information for a tabulated comparison of these values to those of the parent dyes.